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Molecular Cloning of a Novel Ubiquitin-specific Protease, UBP41, with Isopeptidase Activity in Chick Skeletal Muscle*

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A cDNA encoding a new ubiquitin-specific protease, UBP41, in chick skeletal muscle was cloned using an *Escherichia coli*-based *in vivo* screening method. Nucleotide sequence analysis of the cDNA containing an open reading frame of 1,071 base pairs revealed that the protease consists of 357 residues with a calculated molecular mass of 40,847 Da, and is related to members of the UBP family containing highly conserved Cys and His domains. Chick UBP41 was expressed in *E. coli* and purified from the cells to apparent homogeneity, using ^{125}I-labeled ubiquitin-αNH-MHISPPEPESEEEEEHYC as a substrate. The purified enzyme behaved as an approximately 43-kDa protein under both denaturing and nondenaturing conditions, suggesting that it consists of a single polypeptide chain. Like other deubiquitinating enzymes, it was sensitive to inhibition by ubiquitin-aldehyde and sulfhydryl blocking agents, such as *N*-ethylmaleimide. The UBP41 protease cleaved at the C terminus of the ubiquitin moiety in natural and engineered fusions irrespective of their sizes; thus, it is active against ubiquitin-β-galactosidase as well as ubiquitin C-terminal extension protein of 80 amino acids. UBP41 also released free ubiquitin from poly-His-tagged diubiquitin. Moreover, it converted poly-ubiquitinated lysozyme conjugates to mono-ubiquitinated forms of about 24 kDa, although the latter molecules were not further degraded to free ubiquitin and lysozyme. These results suggest that UBP41 may play an important role in the recycling of ubiquitin by hydrolysis of branched poly-ubiquitin chains generated by the action of 26 S proteasome on poly-ubiquitinated protein substrates, as well as in the production of free ubiquitin from linear poly-ubiquitin chains and of certain ribosomal proteins from ubiquitin fusion proteins.

Ubiquitin (Ub)[1] is a highly conserved 76-amino acid polypeptide involved in a variety of cellular functions, including regulation of intracellular protein breakdown, cell cycle regulation, and stress response (1–6). This small protein is covalently ligated to target proteins by a family of Ub-conjugating enzymes, called E2s (7, 8), through an isopeptide linkage between the C-terminal Gly residue of Ub and the ε-amino group of Lys residue(s) of the proteins. Ubs by themselves or in conjugation to proteins may also be ligated to additional Ub molecules to form branched poly-Ub by the linkage between the ε-amino group of Lys-48 of one Ub and the C terminus of the other. Proteins ligated to multiple units of Ub are degraded by the ATP-dependent 26 S proteasome (1–3, 9).

Ubs are encoded by two distinct classes of genes, neither of which encodes a monomeric form of Ub (10, 11). One is a poly-Ub gene, which encodes a linear polymer of Ubs that are linked through peptide bonds between the C-terminal Gly and N-terminal Met of contiguous Ub molecules. The other encodes a fusion protein in which a single Ub is linked to a ribosomal protein consisting of 52 or 76–80 amino acids (12). Thus, generation of free Ub from the linear poly-Ub and Ub fusion proteins and recycling of Ub from the branched poly-Ub ligated to proteins should be essential for Ub-requiring processes, such as intracellular ATP-dependent proteolysis.

A number of Ub C-terminal hydrolases (UCHs) that release Ub molecules that are conjugated to proteins by αNH-peptide bonds or εNH-isopeptide linkages have been identified from different organisms. *Saccharomyces cerevisiae* contains at least five different UCHs, including yeast Ub hydrolase 1 (YUH1) and Ub-specific proteases (UBPs) 1–4 (13–16). Of these, UBP3 has recently been shown to regulate silencing by interaction with SIR4, which is required for silencing of transcription at the silent mating type loci and at telomeres in yeast (17). A family of UCHs, including L1, L2, L3, and H2, has been identified from bovine calf thymus (18–20). Isopeptidase T in human red blood cells has been purified, and its cDNA has been isolated (21, 22). Moreover, it has been shown recently that the 19 S regulatory complex of the 26 S proteasome has intrinsic isopeptidase activity (23).

In addition, the *fat facets* gene in *Drosophila*, which is required in eye development, and the *DUB-1* gene in murine pro-B cell line, which is specifically induced by interleukin-3 and involved in growth suppression, have been shown to encode UCHs (24, 25). We also have identified at least 10 different UCHs in chick skeletal muscle using Ub-αNH-MHISPPEPESEEEEEHYC (referred to as Ub-PESTc) (26–28). Because the Tyr residue next to the C-terminal Cys can be exclusively

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF0167107.

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[1] The abbreviations used are: Ub, ubiquitin; UCH, ubiquitin C-terminal hydrolase; YUH1, yeast ubiquitin hydrolase 1; β-gal, β-galactosidase; UBP, ubiquitin-specific protease; Ub-PESTc, ubiquitin-αNH-MHISPPEPESEEEEEHYC; CEP80, carboxyl extension protein 80; DHFR, dihydrofolate reductase; His-di-Ub, poly-His-tagged di-Ub; Tricine, *N*-tris(hydroxymethyl)methylglycine.

radioiodinated, we could assay the UCH activity by a simple measurement of the radioactivity of the peptide portion that is released into acid-soluble products. Of these, UCH-1 (29), UCH-6 (27), and UCH-8 (30) have been purified and characterized of their biochemical properties.

In eukaryotes, Ub fusion proteins, such as Ub-β-galactosidase (Ub-β-gal), are hydrolyzed by UBPs irrespective of the identity of the residue at the Ub-β-gal junction (with an exception of Pro), making it possible to expose *in vivo* different residues at the N termini of β-gal (31, 32). This technique, required for detection and analysis of the N-end rule in eukaryotes, has been made applicable in bacteria, such as for isolation of the yeast *UBP2* and *UBP3* genes (15). Using a similar approach (*i.e. Escherichia coli*-based *in vivo* screening method), we isolated a cDNA for a new UBP from the cDNA library of chick skeletal muscle in the present study. We also expressed the enzyme in *E. coli*, purified it from the cells, and analyzed its biochemical properties.

EXPERIMENTAL PROCEDURES

Materials—Butyl-Toyopearl was obtained from Tosoh Corp. (Tokyo, Japan), and anti-β-gal antiserum was from Cappel. Ub-αNH-MHISPPEPESEEEEEHYC (Ub-PESTc), Ub-αNH-carboxyl extension protein of 80 amino acids (Ub-CEP80), Ub-αNH-dihydrofolate reductase (Ub-DHFR), and poly-His-tagged di-Ub (His-di-Ub) were purified as described previously (27). The purified Ub-PESTc was radiolabeled with $Na^{125}I$ using IODO-BEADS (Pierce) (33). Ub-aldehyde was prepared by borohydride reduction of Ub in the presence of YUH1 as described elsewhere (34). Reticulocytes were obtained by subcutaneous injections of phenylhydrazine to albino rabbits (35). From the cells, fraction II containing an Ub-conjugating system was prepared as described previously (36).

The chick cDNAs in pBluescript II (pBS) were excised from the chick skeletal muscle λ ZAPII cDNA library (Stratagene) by following the procedure recommended by the manufacturer. The cDNA plasmids were propagated in *E. coli* JM101 cells (*supE thi* Δ*(lac-proAB)/F 39 traΔ36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZΔM15*) (37) that had been transformed with pACUb-R-β-gal or pACUb-M-β-gal (14, 31).

Cloning of Ubp41—The *E. coli* JM101 cells carrying both the chick cDNA plasmids and pACUb-R-β-gal were plated onto Luria broth containing 100 μg/ml ampicillin, 34 μg/ml chloramphenicol, and 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside. They were then incubated at 37 °C for 14 h. Of 10^5 transformants, three appeared as white colonies and the others were stained as blue. All the control cells (*i.e.* transformed with pBluescript II vector only) developed a blue color. From the three white colonies, plasmids were isolated and again transformed into the *E. coli* JM101 expressing either Ub-Arg-β-gal or Ub-Met-β-gal. Only one of the three colonies turned into white when transformed with Ub-Arg-β-gal but not with Ub-Met-β-gal (*i.e.* became blue), confirming that the white colony contains the plasmid carrying the chick cDNA specifying the UBP activity against the Ub-β-gal proteins. The plasmids were isolated and referred to as pBS/Ubp41.

DNA Manipulation and Sequencing—Standard procedures involving recombinant DNA were carried out as described by Ausubel *et al.* (37). DNA fragments produced by restriction endonucleases were subcloned into pBS vector and sequenced using Sanger dideoxy techniques (TaqTrack sequencing system, Promega) and an automatic sequencer (Applied Biosystems model 373A). All sequences were determined on both DNA strands, and all restriction sites used for subcloning were verified as well.

Expression of UBP41 in E. coli—The parental *E. coli* JM101 cells were transformed with pBS/Ubp41 and grown to a late exponential phase in Luria broth containing ampicillin at 37 °C. The cultures were then treated with isopropyl-1-thio-β-D-galactopyranoside to 2 mM, and incubated for another 3 h. After incubation, the cells were collected, resuspended in buffer A (25 mM Tris-HCl buffer (pH 7.8) containing 5 mM 2-mercaptoethanol, 1 mM EDTA, and 10% (v/v) glycerol), and disrupted by French press at 14,000 p.s.i. The cell suspension was centrifuged at 100,000 × *g* for 2 h, and the resulting supernatants were referred to as crude extracts.

In Vitro Assay for UBP Activity—For the *in vitro* assays, ^{125}I-labeled Ub-PESTc was used as a substrate (27). Reaction mixtures (0.1 ml) contained the proper amount of the enzyme preparations and 10–30 μg of ^{125}I-labeled Ub-PESTc in 100 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol. After incubating the mixtures for appropriate periods, the reaction was terminated by adding 50 μl of 40% (w/v) trichloroacetic acid and 50 μl of 1.2% (w/v) bovine serum albumin. The samples were centrifuged, and the resulting supernatants were counted for their radioactivities using a γ-counter. The enzyme activity was expressed as a percentage of ^{125}I-labeled Ub-PESTc hydrolyzed to acid-soluble products. When assaying the hydrolysis of Ub-αNH-carboxyl extension proteins and His-di-Ub, incubations were performed as above but in the presence of 5 μg of the substrate. After incubation for appropriate periods, the samples were subjected to discontinuous gel electrophoresis (see below). Proteins in the gels were then visualized by staining with Coomassie Blue R-250 or by exposing to x-ray films (Fuji) at −70 °C.

Preparation of Poly-ubiquitinated Lysozyme Conjugates—To prepare ^{125}I-labeled poly-Ub-εNH-lysozyme conjugates, 2 μg of the ^{125}I-labeled lysozyme (5×10^5 cpm) was incubated with 10 μg of Ub, 120 μg of fraction II, and an ATP-regenerating system consisting of 10 mM Tris-HCl (pH 7.8), 15 units/ml creatine phosphokinase, 6.5 mM phosphocreatine, 1.5 mM ATP, 1 mM dithiothreitol, 0.5 mM $MgCl_2$, and 1 mM KCl in a final volume of 0.05 ml (36). Incubations were performed for 2 h at 37 °C in the presence of 1 mM hemin to prevent proteolysis of the ubiquitinated protein conjugates by the 26 S proteasome. After incubation, the samples were heated for 10 min at 55 °C for inactivation of endogenous UBPs.

Electrophoresis—Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol was performed as described by Laemmli (38) or using Tris-Tricine buffer as described by Schägger and von Jagow (39). The discontinuous slab gels contained 4, 10, and 16% (w/v) polyacrylamide to improve resolution of small proteins. The sample buffer contained 150 mM Tris-HCl (pH 6.8), 1.5% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromphenol blue, and 7% glycerol. After electrophoresis, the gels were stained with Coomassie Blue R-250 or autoradiographed.

RESULTS

Cloning of Ubp41—To isolate the cDNAs encoding chick muscle UBPs, we took advantage of the facts that *E. coli* lacks both Ub and UBPs, and that the N-end rule operates in *E. coli* as well (14). The finding that Arg-β-gal, but not Ub-Arg-β-gal, is short-lived in *E. coli*, whereas both Met-β-gal and Ub-Met-β-gal are long-lived (14, 31), also made possible the *E. coli*-based *in vivo* screening for UBPs. Specifically, *E. coli* cells expressing the long-lived Ub-Arg-β-gal or Ub-Met-β-gal would form blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactoside plates. If a deubiquitinating activity is also present in the cells, Ub-Arg-β-gal should be converted into the short-lived Arg-β-gal (14), keeping the colonies a white color on the plates. In contrast, the long-lived Met-β-gal generated by the enzyme activity should remain active against 5-bromo-4-chloro-3-indolyl-β-D-galactoside and turn the cells a blue color. Using this double-screening method, we isolated a positive colony that carried a chick muscle cDNA expressing the deubiquitinating activity.

The open reading frame specifying the deubiquitinating activity was identified by subcloning and nucleotide sequencing of the cDNA, and named *Ubp41*, because it encodes a 41-kDa UBP (see below). The flanking nucleotide sequence around the initiation codon, GTTATGG, exhibits a G at −3 and +4 (Kozak consensus sequence), without which ATG is bypassed (40). The position of the start (ATG) codon was inferred to yield the 1,071-base pair open reading frame, which encodes a protein consisting of 357 residues (40,847 Da) (Fig. 1). From the deduced amino acid sequence, the isoelectric point of UBP41 was estimated to be 9.01. In addition, sequence alignment demonstrated the similarity of UBP41 with several deubiquitinating enzymes, including human tre-2 (41), mouse DUB-1 (25), and yeast Doa4 (16). However, the sequence similarity was largely restricted to the conserved Cys and His domains that are characteristic of the UBP family (Fig. 2).

Purification of UBP41—To purify UBP41, crude extracts (14 g of protein) were prepared from 175 g of *E. coli* cells that had been transformed with pBS/Ubp41. The activity of UBP41 in

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      GAATTCCCGTGTCCAGCCCCTCCGGCAGCCAGCTCTGTGTAAAGGGATGATGGTT  (-55)
ATGGCGGCACGCATGGCCCCGACCCCACGCAGCTCCAAGGTGGTGCAGGGGCTGACTGGG    60
  M  A  A  R  M  A  P  T  P  R  S  S  K  V  V  Q  G  L  T  G     20
CTGCGGAACCTCGGCAATACGTGCTTCATGAACTCCATCCTGCAGTGCCTGAGCAACACC   120
  L  R  N  L  G  N  T  C  F  M  N  S  I  L  Q  C  L  S  N  T     40
AAGGAGCTGCGGGATTACTGCCTGCAGAACCAGTACCTGCGGGACCTCAACAACAACAGC   180
  K  E  L  R  D  Y  C  L  Q  N  Q  Y  L  R  D  L  N  N  N  S     60
CGCATGCGCACCGCGCTCATGTCAGAGTTTGCAAAGCTGATCCAGCTGCTCTGGACCTCA   240
  R  M  R  T  A  L  M  S  E  F  A  K  L  I  Q  L  L  W  T  S     80
TCCCCCAATGACAGCGTGAGTCCCTCCGAGTTCAAGACGCAGATCCAGAGATACGCCCCA   300
  S  P  N  D  S  V  S  P  S  E  F  K  T  Q  I  Q  R  Y  A  P    100
CGCTTTGTTGGATACAACCAGCAGGATGCACAGGAGTTCCTGCGCTTCCTCCTGGATGGG   360
  R  F  V  G  Y  N  Q  Q  D  A  Q  E  F  L  R  F  L  L  D  G    120
CTGCACGGCGAGGTGAACCGCGTGCTGGTGCGGCCGCGGGCCAATGCTGACACCTTGGAC   420
  L  H  G  E  V  N  R  V  L  V  R  P  R  A  N  A  D  T  L  D    140
CACCTCCCTGATGATGAGAAGAGCCGCCAGATGTGGCGGAGGTACCAGGAGCGGGAGGAC   480
  H  L  P  D  D  E  K  S  R  Q  M  W  R  R  Y  Q  E  R  E  D    160
AGTCGTGTTAGTGATCTCTTCGTTGGGCAGCTGAAGAGCTCACTGACCTGCAGTGAGTGT   540
  S  R  V  S  D  L  F  V  G  Q  L  K  S  S  L  T  C  S  E  C    180
GGCTACTGCTCCACAGCCTTTGACCCCTTCTGGGACCTCTCCCTGCCCATCCCCAAGAAA   600
  G  Y  C  S  T  A  F  D  P  F  W  D  L  S  L  P  I  P  K  K    200
GGCTATGGGGAAGTGACCCTGATGGACTGCCTGCGGCTCTTCACCAAAGAGGACGTTCTG   660
  G  Y  G  E  V  T  L  M  D  C  L  R  L  F  T  K  E  D  V  L    220
GATGGGGATGAGAAACCGACGTGCTGTCGCTGCAAAGCCAGGACGCGATGCACCAAGAAA   720
  D  G  D  E  K  P  T  C  C  R  C  K  A  R  T  R  C  T  K  K    240
TTCAGCATCCAGAAGTTCCCCAAGATCCTGGTGCTCCACCTGAAGCGCTTCTCAGAGGCC   780
  F  S  I  Q  K  F  P  K  I  L  V  L  H  L  K  R  F  S  E  A    260
AGGATACGAGCCAGCAAGCTCACCACCTTCGTCAACTTCCCACTGAAGGACCTGGACCTG   840
  R  I  R  A  S  K  L  T  T  F  V  N  F  P  L  K  D  L  D  L    280
AGGGAGTTCGCCTCGCAGAGCTGCAACCACGCCGTTTACAACCTCTACGCCGTCTCTAAC   900
  R  E  F  A  S  Q  S  C  N  H  A  V  Y  N  L  Y  A  V  S  N    300
CACTCGGGCACCACCATGGGGGGACACTACACTGCCTACTGCAAGAGCCCCATCTCCAGC   960
  H  S  G  T  T  M  G  G  H  Y  T  A  Y  C  K  S  P  I  S  S    320
GAGTGGCACAGCTTCAACGATTCCCGCGTCACCCCCATGTCCTCCAGCCACGTGCGCAGC  1020
  E  W  H  S  F  N  D  S  R  V  T  P  M  S  S  S  H  V  R  S    340
AGCGATGCCTACCTGCTCTTCTACGAGCTGGCCAGCCCATCCTCACGCATGTAGCCGGCC  1080
  S  D  A  Y  L  L  F  Y  E  L  A  S  P  S  S  R  M  *         357
CCCGGGCACCCCTCGGAGCCTCCTACCTGGATTTGGGCCCCCCAAGAACAGCAGAAAGCC  1140
CCGACGATGGCAGGACCCAGGCAGCCCCAGTGCCTCCGGGCCGCTGGCGTCTGCTTTT  1200
TACCCTGGGGTAGGTTTTTCTTTTTCTCTTTCTGGTGTGTAATAAAAATACTGAGCAAGG  1260
GCCGTGGGGCTGCCTGCTCCCACCGGGACCTCGGGGACACCCGGAGTTGGGTCTCTTCTG  1320
GCATCTCCGTACCACTCTGCCATCCCCGAGGATCCGTGAGCCTCCTCCCAGCTCCTCCAA  1380
ACCTCCTGGTGAAGGATCGCAGCTCTTCGGTAGCCTCCATCCCACCTCCATCCTCGTTCC  1440
GATCGGCCCCAGACTCACGGCCAGGAGGGAGGATGATCCTTCATGGAGTTTCTTTCTATT  1500
TATCACGGACTTCTCTCAGCCCGGACCATCCCTCCTGGCTGCCCAGGGACTCAGGCCGGG  1560
GGGGGCTGACCCACCTCCTTATACGACGGTGCCTTAAACCATTTAGGCGCGCAGGAGGCC  1620
CTTGGGGTGCACAGGGATGTCCGTCCTACGGGGGGGTGGGGGGGTGGGCAGCACCTTTCT  1680
AGTAGTGGGGCTTTCCTCCCCTCTTCACCAGGAGCCCGGAATTC                  1724
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FIG. 1. **Nucleotide sequence of the *Ubp41* cDNA and deduced amino acid sequence of the UBP41 protein.** The nucleotides are numbered on the *right*, beginning at the A of the presumed start codon. The amino acid residues are also numbered on the *right*. The *asterisk* indicates the TAG stop codon.

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                    Cys Domain

Chick UBP41   20 GLRNLGNTCFMNSILQCL 37
Human tre-2  216 GLGNLGNTCFMNSALQCL 233
Mouse DUB-1   52 GLQNTGNSCYLNAALQCL 69
Yeast Doa4    56 GLENLGNSCYMNCIIQCI 580

                    His Domain

Chick UBP41  293 YNLYAVSNHSGTTM-GGHYTAYCK SPISSEWH 323
Human tre-2  995 YNLYAISCHSGILS-GGHYITYAK NP-NCKWH 1024
Mouse DUB-1  290 YALYAVLVHDGATSHS GHYFCCVK AG-HGKWY 320
Yeast Doa4   864 YRLYGVACHFGTLY-GGHYTAYVK KGLKKGWL 894
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FIG. 2. **Sequence similarity among UBP family.** Sequence homology between chick UBP41 (this work), human tre-2 (42), murine DUB-1 (25), and yeast Doa4 (16) is shown. Identical amino acid residues in the regions containing the conserved Cys and His domains are *boxed*. The first and last residues of each sequence are indicated by the *numbers*.

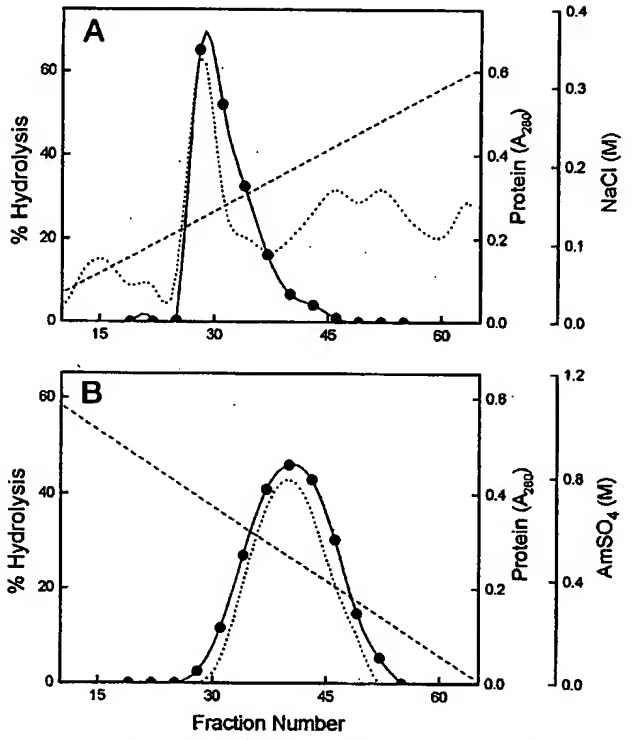

FIG. 3. **Purification of UBP41 from *E. coli*.** *A*, the UBP41 preparation obtained from the DEAE-Sepharose column was subjected to chromatography on a S-Sepharose column as described under "Results." Fractions of 10 ml were collected, and aliquots (2 μl) of them were assayed for their ability to hydrolyze ^{125}I-labeled Ub-PESTc (10 μg) (●) as described under "Experimental Procedures." Incubations were performed for 15 min at 37 °C. The *dashed line* shows the linear gradient of NaCl, and the *dotted line* indicates the protein profile. *B*, the enzyme preparation obtained from the phosphocellulose column was chromatographed using a butyl-Toyopearl column as described under "Results." Fractions of 0.5 ml were collected, and aliquots (2 μl) of them were assayed as above. The *dashed line* shows the reverse gradient of ammonium sulfate.

chromatographic fractions obtained during purification was monitored by determining its ability to hydrolyze ^{125}I-labeled Ub-PESTc. The extracts were applied to a DEAE-Sepharose column (2.5 × 19 cm) equilibrated with buffer A (pH 7.8). The DEAE flow-through fractions were pooled and loaded onto a S-Sepharose column (2.5 × 19 cm) equilibrated with the same buffer. After washing the column, proteins bound to the column were eluted with a linear gradient of 0.1–0.4 M NaCl (Fig. 3*A*). Fractions with high activity were pooled, added with the same volume of buffer A containing 2.4 M ammonium sulfate, and applied to a phenyl-Sepharose column (1 × 4 cm). After washing with buffer A containing 1.2 M ammonium sulfate, proteins bound to column were eluted with a reverse gradient of ammonium sulfate from 1.2 M to 0 M. The active fractions were pooled, dialyzed against buffer B (20 mM NaH_2PO_4/Na_2HPO_4, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 10% glycerol, pH 7.8), and loaded onto a phosphocellulose column (1 × 4 cm). After washing with the same buffer, proteins bound to the column were eluted with a linear gradient of 20–300 mM phosphate. The active fractions were pooled, dialyzed against buffer A containing 1.2 M ammonium sulfate, and loaded onto a butyl-Toyopearl column (1 × 2.5 cm). After washing with the same buffer, proteins bound were eluted with a reverse gradient of ammonium sulfate from 1.2 M to 0 M. Fractions under the symmetric peak of the Ub-PESTc-degrading activity (Fig. 3*B*) were pooled, dialyzed against buffer A, concentrated by ultrafiltration, and kept frozen at −70 °C until use. A summary of the purification of UBP41 is shown in Table I.

The size of the enzyme was estimated to be about 43 kDa upon chromatography on Superose-12 column (1 × 30 cm) equilibrated with buffer A containing 0.1 M NaCl (Fig. 4*A*). In addition, it ran as a single protein of 43 kDa upon analysis by polyacrylamide gel electrophoresis in the presence of SDS and 2-mercaptoethanol (Fig. 4*B*). Because the enzyme was expressed as a fusion protein, it ran slightly larger than 41 kDa as expected under both nondenaturing and denaturing conditions. These results indicate that UBP41 comprises a single polypeptide.

Biochemical Properties—To determine the time-dependent hydrolysis of ^{125}I-labeled Ub-PESTc by the purified UBP41, assays were performed by incubation of the enzyme for various

TABLE I
Summary of purification of UBP41

Hydrolysis of ^{125}I-labeled Ub-PESTc was assayed by incubation of 30 μg of the substrate with aliquots of the proteins obtained from each purification steps for 15 min at 37 °C as in Fig. 5.

Steps	Protein	Total activity	Specific activity
	mg	*units*[a]	*units/mg*
Crude extract	14,000	5,610	0.4
DEAE-Sepharose	1,680	4,692	2.8
S-Sepharose	25.5	1,112	44
Phenyl-Sepharose	2.0	92	46
Phosphocellulose	0.32	31	97
Butyl-Toyopearl	0.07	7.7	110

[a] One unit was defined as 1 mg of Ub-PESTc hydrolyzed to acid-soluble products/h.

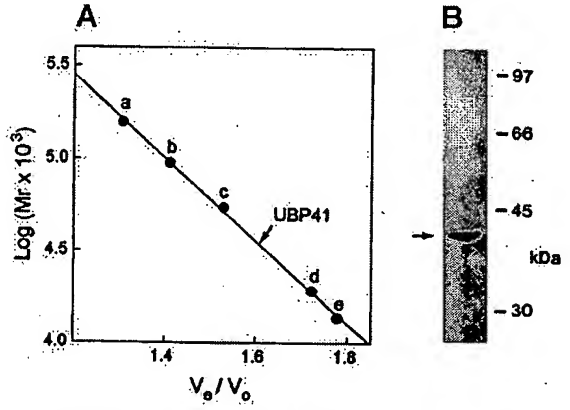

FIG. 4. **Estimation of the size of UBP41.** *A*, the purified enzyme (10 μg) was chromatographed on a Superose-12 column (1 × 30 cm) equilibrated with buffer A containing 0.1 M NaCl. Fractions of 0.5 ml were collected, and aliquots (5 μl) of them were assayed for their ability to hydrolyze ^{125}I-labeled Ub-PESTc. The size markers used are: *a*, catalyase (232 kDa); *b*, aldolase (158 kDa); *c*, bovine serum albumin (66 kDa); *d*, chymotrypsinogen A (25 kDa); *e*, ribonuclease A (13.7 kDa). *B*, the enzyme (5 μg) was also electrophoresed on a 11% polyacrylamide slab gel containing SDS and 2-mercaptoethanol as described by Laemmli (38). Proteins in the gel were then visualized by staining with Coomassie Blue R-250. The *arrow* indicates the UBP41 band.

periods at 37 °C. Fig. 5*A* shows that the enzyme activity increases linearly with time for at least 30 min. We then examined the effects of increasing concentrations of the substrate on the enzyme activity (Fig. 5*B*). Upon a double-reciprocal plot of the data, the K_m of Ub-PESTc for its cleavage was estimated to be about 12 μM.

We also examined the effects of sulfhydryl blocking agents, such as *N*-ethylmaleimide and Ub-aldehyde, both of which are known to inhibit the activities of all UCHs and UBPs so far identified (42, 43). *N*-Ethylmaleimide at 1 mM almost completely blocked the activity of UBP41 against Ub-PESTc (Fig. 5*A*). Ub-aldehyde also strongly inhibited the enzyme activity with a K_i of about 0.7 μM (Fig. 5*B*). However, the metal-chelating agents, including *o*-phenanthroline (1 mM), or the inhibitors of serine proteases, such as phenylmethylsulfonyl fluoride (1 mM), showed little or no effect (data not shown). Nor was the enzyme activity influenced by poly-L-Lys (data not shown), which is known to stimulate dramatically the Ub-PESTc-degrading activity of YUH1 as well as of most UCHs identified in chick skeletal muscle (27).

Substrate Specificity—To determine the substrate specificity of UBP41, the purified enzyme was incubated with various Ub-αNH-protein extensions, such as Ub-DHFR, Ub-CEP80, and His-di-Ub. After incubation, the samples were subjected to discontinuous gel electrophoresis under denaturing conditions, followed by staining with Coomassie Blue R-250. Assays were

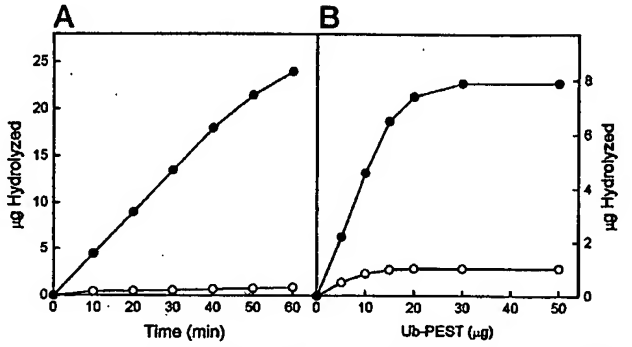

FIG. 5. **Hydrolysis of ^{125}I-labeled Ub-PESTc by the purified UBP41.** *A*, reaction mixtures contained 50 ng of the purified UBP41 and 30 μg of ^{125}I-labeled Ub-PESTc (0.5–1 × 10^3 cpm/μg) in 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol. The mixtures were incubated for various periods at 37 °C in the absence (●) and presence of 1 mM *N*-ethylmaleimide (○). *B*, assays were performed as above but by incubation of the enzyme for 15 min in the presence of increasing concentrations of the substrate and without (●) and with 1 μM Ub-aldehyde (○).

also performed with Ub-PESTc as a control. As shown in Fig. 6*A*, UBP41 was capable of generating free Ub from all of the substrates tested. To confirm whether Ub-Met-β-gal can also be hydrolyzed *in vitro* by the purified UBP41, the enzyme was incubated with the extracts of *E. coli* JM101 cells transformed with pACUb-M-β-gal and then with anti-β-gal antibody for immunoblot analysis. Fig. 6*B* shows that the purified enzyme can release β-gal from Ub-Met-β-gal expressed in the *E. coli* extracts.

To determine whether the purified UBP41 can also act on branched poly-Ub chains, poly-Ub-εNH-lysozyme conjugates were prepared by incubation of ^{125}I-labeled lysozyme and reticulocyte lysate fraction II. The radiolabeled poly-Ub-lysozyme conjugates were then incubated with the purified UBP41. Upon incubation, the amount of high molecular weight conjugates significantly decreased with a concomitant increase in that of a 24-kDa band, which corresponds approximately to the size of mono-ubiquitinated lysozyme, in addition to a 42-kDa conjugate (Fig. 7). Treatment of *N*-ethylmaleimide strongly inhibited the generation of both 24-kDa and 42-kDa conjugates. In addition, prolonged incubation with higher amounts of UBP41 resulted in an additional increase in the level of the 24-kDa band (data not shown). Thus, it appears that UBP41 can release Ubs that are conjugated to proteins or adjacent Ub molecules by εNH-isopeptide linkages as well as by αNH-peptide bonds, except from the singly ubiquitinated lysozyme molecule.

DISCUSSION

In the present study, we have cloned a cDNA that encodes a 41-kDa UBP in chick skeletal muscle. The 357-residue protein has no significant sequence similarity to previously isolated yeast or mammalian UBPs, except for two short regions containing Cys and His domains. Wilkinson *et al.* (43) have recently reported that the UBP family contains a minimal catalytic core, which spans the sequence between the active site Cys and the His box, and ranges in the number of amino acids from about 300 to 840 residues. The minimal catalytic domain of UBP41, which is composed of 330 residues, is also within this range. In addition, the UBP family has N- or C-terminal extensions, which seem to control substrate specificity, protein-protein interactions, and localization. For instance, human isopeptidase T has a N-terminal extension of 300 amino acids, whereas mouse DUB-1 has a C-terminal extension of 181 amino acids. Interestingly, UBP41 has neither the N- or the C-terminal extension, making it the smallest functional UBP identified to date.

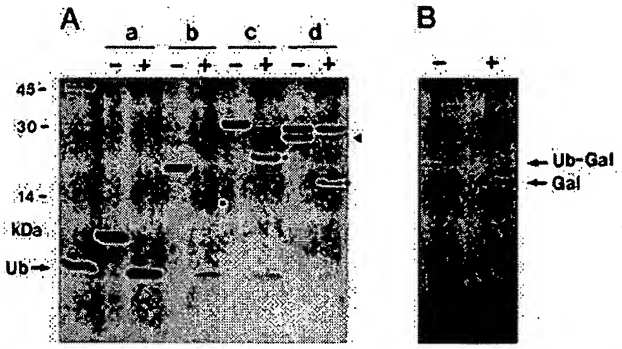

FIG. 6. **Hydrolysis of various Ub-αNH-protein extensions and Ub-Met-β-gal by UBP41.** *A*, aliquots (5 μg each) of the purified Ub-PESTc (*lanes a*), Ub-CEP80 (*lanes b*), Ub-DHFR (*lanes c*), and His-di-Ub (*lanes d*) were incubated alone (− *lanes*) or with 100 ng of UBP41 (+ *lanes*) for 2 h at 37 °C. After incubation, the samples were subjected to discontinuous gel electrophoresis under denaturing conditions as described under "Experimental Procedures." Proteins in the gels were then visualized by staining with Coomassie Blue R-250. The *arrowhead* indicates His-di-Ub. The protein band seen just above His-di-Ub (in *lanes d*) is an unknown protein contaminated during preparation of the substrate. *B*, the extracts (50 μg) obtained from *E. coli* JM101 cells expressing Ub-Met-β-gal was incubated in the absence (− *lane*) and presence of 100 ng of UBP41 (+ *lane*) for 2 h at 37 °C. After incubation, the samples were electrophoresed as above and then subjected to immunoblot analysis using anti-β-gal antibody.

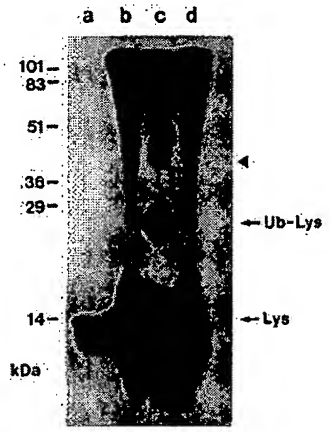

FIG. 7. **Hydrolysis of the branched poly-Ub chains by UBP41.** Poly-Ub protein conjugates were prepared by incubating ^{125}I-labeled lysozyme and reticulocyte lysate fraction II. The radiolabeled lysozyme conjugates were then incubated alone (*lane b*) or with 100 ng of UBP41 in the absence (*lane c*) and presence of 1 mM *N*-ethylmaleimide (*lane d*) for 2 h at 37 °C. After incubation, the samples were subjected to discontinuous gel electrophoresis under denaturing conditions followed by autoradiography. *Lane a* indicates ^{125}I-labeled lysozyme incubated alone. *Lys* and *Ub-Lys* denote free ^{125}I-labeled lysozyme and mono-ubiquitinated lysozyme, respectively. The *arrowhead* indicates an additional, 42-kDa product.

We also have demonstrated that the purified UBP41 hydrolyzes not only Ub-αNH-extension proteins, such as Ub-CEP80, but also His-di-Ub. These results suggest that UBP41 may play an important role in the generation of free Ubs from the linear polymer of Ubs and in the production of free Ubs and certain ribosomal proteins from their conjugates. UBP41 is also capable of releasing free Ubs from branched poly-Ub chains, suggesting that the enzyme may also function in the recycling of Ubs in muscle cells, perhaps after degradation of poly-Ub-protein conjugates by the 26 S proteasomes.

Of interest is the finding that UBP41 can rapidly deubiquitinate from branched poly-Ub chains and accumulate the 24-kDa mono-Ub-εNH-lysozyme conjugates (see Fig. 7). Incubation of poly-Ub-lysozyme conjugates for a prolonged period in the presence of increasing amounts of the enzyme further accumulated the mono-Ub-lysozyme (data not shown). This finding was rather surprising, because a lysozyme molecule contains six Lys residues, all of which can be ubiquitinated by forming isopeptide bonds, although perhaps to different extents. Moreover, it has been demonstrated previously that lysozyme is ubiquitinated at multiple sites (44). Therefore, we did not expect that UBP41 accumulates a single species of mono-Ub-lysozyme conjugate, in which only one of the six Lys residues in lysozyme is ligated to an Ub molecule. One possible explanation may be that UBP41 may also be capable of removing Ub from the lysozyme molecules that are mono-ubiquitinated at multiple Lys sites, but not from the molecules that are mono-ubiquitinated at a certain, specific Lys site. In the mono-Ub-lysozyme molecule, the Gly-Lys bond may be sterically shielded from the attack by UBP41.

Previously, we have identified at least 10 different UCH activities from the extracts of chick skeletal muscle, and we have tentatively named these UCH-1 to UCH-10 (27). Of these, UCH-1, UCH-6, and UCH-8 have been purified and their biochemical properties have been characterized (27, 29, 30). In a number of criteria, however, UBP41 appears distinct from the previously isolated UCHs. First, the activity of UBP41 is not at all influenced by the treatment of poly-L-Lys, unlike the chick UCHs, except UCH-1, whose activities are dramatically stimulated the polycationic agent. Second, UBP41, but not UCH-1, is capable of converting *in vitro* the Ub-β-gal proteins into Ub and β-gal, although both are active against Ub-extension proteins with relatively low molecular masses, such as Ub-CEP80 and Ub-DHFR. Third, the molecular mass of UBP41 is different from that of UCH-1, which is 34 kDa. Fourth, the specific activity of UBP41 is much higher than that of UCH-1. Finally, the chromatographic behavior of UBP41 during purification differs from that of other chick UCHs. Thus, UBP41 must be a novel protease.

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